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The goal of this research is to understand the regulation of Ras-mediated signaling in *C. elegans* vulval development. We describe the identification and characterization of a novel gene, *sur-8*, that functions to regulate a receptor tyrosine kinase-Ras-MAP kinase-mediated signal transduction pathway during *C. elegans* vulval development. Mutations in *sur-8* were identified as suppressors of an activated *let-60 ras* mutation. Our genetic analysis indicates that *sur-8* plays a positive regulatory role in Ras-mediated signaling, and appears to function downstream of Ras but not downstream of Raf. Although *sur-8* mutations by themselves have no effect on normal Ras-mediated signaling, reduction of *sur-8* function dramatically enhances *mpk-1* MAP kinase and *ksr-1* mutations and an increase of *sur-8* dosage enhances an activated *let-60 ras* mutation. We found that *sur-8* encodes a novel protein conserved in mammals that is composed predominantly of leucine-rich repeats. SUR-8 interacts directly with LET-60 Ras, but fails to interact with a putative effector domain mutant, P34G. A structural and functional SUR-8 homologue in humans specifically binds K-Ras and N-Ras but not H-Ras in vitro. Our results indicate that *sur-8* is an evolutionarily conserved positive regulator of Ras signaling pathways and that SUR-8 may mediate its effects through Ras binding.

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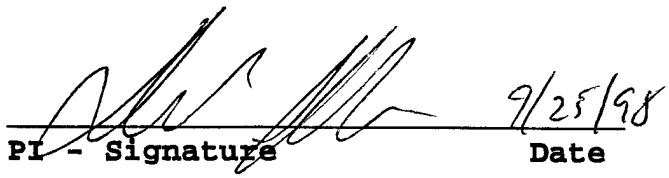

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"Genetic and Molecular Analysis of Suppressors of Ras Mutations"

NOTE: This report is a copy of the manuscript titled: **SUR-8, a conserved Ras-binding protein with leucine-rich repeats, positively regulates Ras-mediated signaling in *C. elegans*.** Figures are not included in this report and can be found in: **Sieburth et al., Cell 94:119-130 (1998).**

INTRODUCTION:

The Ras family of proteins play critical roles in cell proliferation, differentiation and migration in response to extracellular signals. Ras proteins are 21 kD membrane-bound GTPases that act as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. In the best studied Ras-mediated signal transduction pathways, Ras is activated by receptor tyrosine kinases (RTK) through guanine nucleotide exchange factors, which promote GTP binding and a conformational change in Ras to an active state (McCormick, 1993; Schlessinger, 1993). GTP-bound Ras then binds to the serine/threonine kinase Raf and recruits it to the plasma membrane where it is activated. Once activated, Raf phosphorylates and activates the dual specificity kinase MEK, which in turn phosphorylates and activates MAP kinase. Activated MAP kinase is proposed to regulate the activity of multiple targets including transcription factors which mediate a variety of physiological responses (Marshall, 1994).

Biochemical studies using mammalian tissue culture and genetic analysis of *C. elegans* and Drosophila suggest that this RTK-Ras-MAP Kinase signal transduction pathway is not a simple linear pathway but is likely part of a complicated network (Katz and McCormick, 1997; Sundaram and Han, 1996; Wassarman et al., 1995). Two important questions remain to be addressed regarding the relationship between the linear Ras pathway and other factors involved in the signaling process. The first question is related to how activation of this pathway leads to diverse cellular responses. For example, if the Ras pathway is to be regulated at the level of Ras, the same Ras protein may have different upstream regulators as well as different downstream effectors for different functions. In recent years several potential Ras effectors in addition to Raf have been described, including phosphatidylinositol-3-OH (PI-3) kinase and Ral GDS (Katz and McCormick, 1997), and are good candidates for defining branch points for Ras signaling. In addition, different Ras family members may perform distinct cellular functions by associating with unique sets of regulators or effectors.

In mammals, the Ras family is composed of four highly related members, H-Ras, N-Ras, K-Ras 4A and K-Ras 4B (Barbacid, 1987). Family members are 100% identical in the first 86 amino acids which contains the effector domain (amino acids 32-40) and are most divergent in the C-terminal 26 amino acids which contains the lipid modified membrane targeting domains (amino acids 164-189). K-*ras*, N-*ras*, and H-*ras* have widely overlapping spatial and temporal patterns of expression (Furth et al., 1987; Leon et al., 1987), and each family member is found mutated in certain tumor types (Bos, 1988).

Thus, they are thought to have overlapping functions. This notion is supported by studies showing that N-*ras* (Umanoff et al., 1995) or H-*ras* (M. Katsuki, unpublished) deficient mice have no apparent abnormalities. However, K-*ras* knockout mice exhibit embryonic lethality and early hematopoetic defects, phenotypes which are exacerbated by reduction of N-*ras* dosage (Johnson et al., 1997). Thus, even though family members are at least partially functionally redundant, the K-*ras* knockout mice provide evidence for a unique function for K-*ras*. Further support for distinct Ras functions comes from the observation that many tumor types are associated with activating mutations of primarily one particular Ras family member (Leon et al., 1987). It is thus also possible that different Ras family members interact with distinct subsets of proteins that mediate unique regulatory or effector functions.

The second question regarding the complexity of the Ras-mediated signaling processes is what collaborative roles multiple factors and signaling branches may have in regulating the output of the signal. The main components of the RTK-Ras-MAP kinase pathway may be essential elements of a given signaling process, but there may be other factors that feed in to or out of this pathway that play important regulatory functions to ensure the maximal activity of the pathway and to tighten the regulation of the signal. For example, the *ksr* genes were identified as suppressors of activated *ras* in *C. elegans* and Drosophila (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995), and their biochemical relationship with the Ras pathway is still not well understood. In *C. elegans*, it has been shown that mutations in the *ksr-1* gene do not obviously disrupt vulval signal transduction mediated by *ras*. However, the *ksr-1* activity becomes essential when the activity in the main pathway is compromised (Kornfeld et al., 1995; Sundaram and Han, 1995).

C. elegans provides a powerful genetic system to identify and characterize genes that regulate the Ras-mediated signal transduction pathway. In *C. elegans*, there is a single known *ras* gene, *let-60*, that acts in a RTK-Ras-MAP kinase signal transduction pathway to specify several cell fates, including those of the vulva. The hermaphrodite vulva is derived from three of six vulval precursor cells (VPCs). Each of the six VPCs has the ability to adopt either a vulval cell fate or a non-vulval cell fate. The proper pattern of cell fate specification is determined by the combination of three signaling events (Figure 1A) (Kornfeld, 1997; Sundaram and Han, 1996). A signal from the neighboring anchor cell induces three VPCs to adopt vulval cell fates by activating a Ras-mediated signal transduction pathway (Figure 1B). Mutations that reduce signaling activity cause less than three VPCs to adopt a vulval cell fate (Vulvaless, or Vul). Mutations that cause constitutive activation of the pathway (e.g., activated *ras* mutations) cause up to all six VPCs to adopt vulval cell fates (Multivulva or Muv). Many genes in the pathway function in multiple signaling events during *C. elegans* development. For example, *let-60 ras* has also been shown to function in male tail fate specification (Chamberlin and Sternberg, 1994), germ cell and oocyte development (Church et al., 1995; Gutch et al., 1998), sex-myoblast migration (Sundaram et al., 1996) and excretory duct cell fate specification (Yochem et al., 1997).

To identify new factors acting downstream of *let-60 ras*, our laboratory and others have screened for mutations that can suppress activated *let-60 ras* mutations (Kornfeld, 1997; Sundaram and Han, 1996). In addition to identifying genes such as *lin-45 raf*, *mek-2* MEK and *mpk-1* MAP kinase which act in the main pathway downstream of *let-60 ras*, we have identified mutations in a number of new genes which can suppress the Muv phenotype caused by activated *let-60 ras*, but do not cause an obvious vulval phenotype on their own. Here we describe the identification and characterization of one such gene, *sur-8*.

Our genetic analysis indicates that *sur-8* acts downstream of *let-60 ras* but not downstream of *raf* to positively regulate Ras-mediated signaling. Although *sur-8* mutations do not cause a vulval phenotype on their own, *sur-8* function is essential when the activities of either *mpk-1* MAP kinase or *ksr-1* are compromised. We further show that *sur-8* encodes a novel protein that is predominantly composed of leucine-rich repeats (LRRs). Finally, we demonstrate that SUR-8 interacts with LET-60 Ras and that a highly conserved human SUR-8 homologue appears to have binding specificity for K-Ras and N-Ras but not H-Ras.

BODY:

Results

Mutations in *sur-8* Suppress Activated *let-60 ras*

To identify factors that act downstream of Ras during vulval induction, we screened for extragenic suppressor mutations that would revert the Multivulva (Muv) phenotype caused by a gain-of-function *let-60 ras* mutation, *n1046gf*, back to wild type. The *n1046gf* mutation encodes a Gly to Glu substitution at codon 13 (G13E) (Beitel et al., 1990) also found in human Ras oncoproteins (Bos, 1988). By screening for suppressors of this activated *ras* mutation, we expected to identify mutations in genes acting downstream of or in parallel to *let-60 ras* or in genes involved in the proper expression or activation of *let-60 ras*. The parental strain used for screening carries multiple copies of a *let-60 ras(n1046gf)* genomic fragment (Sundaram et al., 1996) and displays a completely penetrant Muv phenotype. This increased penetrance of the Muv phenotype over that caused by non-transgenic *let-60 ras(n1046gf)* animals allowed us to rapidly screen a large number of genomes for suppressor mutations. From 22,000 haploid genomes screened, we isolated 11 mutations in at least four genes, including a single mutation in the *sur-8* locus (suppresor of *ras*), *ku167*, three alleles of *lin-45 raf* and 3 alleles of *mek-2* MEK. We identified a second allele of *sur-8, ku242*, in a non-complementation screen that was not biased against isolating null mutations. *sur-8(ku242)* failed to complement the suppression phenotype of *sur-8(ku167)* in a *let-60 ras(n1046gf)* background.

Both *sur-8(ku167)* and *sur-8(ku242)* mutations suppressed the Muv phenotype caused by *let-60 ras(n1046gf)* to nearly wild type and suppressed the male mating defect associated with *let-60 ras(n1046gf)*. For example, the *sur-8(ku167)* mutation reduced the Muv phenotype of *let-60 ras(n1046gf)* animals from 87% to 4% (Table 1). The suppression observed was due to a decrease in the average vulval induction of the VPCs from 154% to 102% (Table 1). Both *sur-8* mutations most often reverted the pattern of ectopic vulval induction back to a wild type pattern (data not shown). Since *sur-8(ku167)* was a slightly stronger suppressor than *sur-8(ku242)*, further genetic characterization was performed using *sur-8(ku167)*.

Genetic dosage analysis indicated that the *sur-8(ku167)* mutation is a recessive, strong loss-of-function mutation. The deficiency *mDf4* failed to complement *sur-8(ku167)* for the Suppression phenotype. Animals in which *ku167* was in trans to *mDf4*, and thus contained only one copy of *sur-8(ku167)*, displayed a suppression phenotype that was similar to, but slightly stronger than, animals homozygous for *sur-8(ku167)* (Table 1). In addition, the duplication *mDp1*, which covers the *sur-8* locus, reverted the suppression phenotype of *sur-8(ku167) let-60(n1046gf)* animals to 90% Muv (Table 1). Finally, a

mutant *sur-8(ku167)* gene when overexpressed in *sur-8(ku167) let-60(n1046gf)* mutants retained very little but some *sur-8* activity (data not shown). Thus, the *sur-8(ku167)* mutation results in severe reduction but probably not elimination of *sur-8(+)* function.

Table 1. Phenotype and Gene Dosage Analysis of *sur-8* Mutant Animals

#	*sur-8* genotype[a]	*let-60 ras* genotype	phenotype %Muv(n)[b]	phenotype % induction (n)[c]
1	+	+	0 (many)	100 (many)
2	+	*gf*	87 (276)	154 (27)
3	*ku167/ku167*	*gf*	4 (333)	102 (43)
4	*ku167/+*	*gf*	77 (57)	129 (16)
5	*ku242/ku242*	*gf*	7 (328)	103 (33)
6	*ku242/+*	*gf*	84 (175)	153 (15)
7	*ku167/ku242*	*gf*	11 (160)	103 (31)
8	*ku167/ku167; mDp1*	*gf*	90 (200)	154 (32)
9	*+; mDp1*	*gf*	100 (247)	182 (30)
10	*+; kuEx83*	*gf*	100 (200)	196 (18)
11	+	*gf/+*	nd	106 (30)
12	*+; kuEx83*	*gf/+*	nd	145 (28)
13	*ku167/mDf4*[d]	*gf*	14 (270)	108 (32)
14	*+/mDf4*[d]	*gf*	68 (233)	136 (22)

[a] The complete genotypes for each strain are: 1, N2 (wildtype); 2, *let-60(n1046)*; 3, *sur-8(ku167) let-60(n1046)*; 4, *sur-8(ku167) unc-24 let-60(n1046)/+ let-60(sy130) dpy-20* [*sy130* encodes the same G13E substitution as *n1046* (Beitel et al., 1990)]; 5, *sur-8(ku242) let-60(n1046)*; 6, *sur-8(ku242) unc-24 let-60(n1046)/let-60(sy130) dpy-20*; 7, *sur-8(ku167) let-60(n1046)/sur-8(ku242) unc-24 let-60(n1046)*; 8, *sur-8(ku167) unc-5 let-60(n1046); mDp1*; 9, *unc-5 let-60(n1046); mDp1*; 10, *let-60(n1046); kuEx83. kuEx83* is a transgene carrying *sur-8(+)* genomic DNA; 11, non-transgenic *let-60(sy130) dpy-20/unc-24* siblings of 12; 12, *let-60(sy130) dpy-20/unc-24; kuEx83*; 13, *unc-5 sur-8(ku167) let-60(n1046)/dpy-13 mDf4 let-60(n1046);* 14, *let-60(n1046)/let-60(n1046) dpy-13 mDf4.*

[b] Percent Multivulva was determined by scoring adult hermaphrodites for presence of ventral protusions under a dissecting microscope. "n" indicates the number of animals scored. nd, not determined.

[c] Average percentage of VPCs adopting a vulval cell fate per animal. In wild type (100% induction), three of six VPCs are induced.

[d] The *dpy-13* marker is semi-dominant and, when heterozygous, reduces the ability of *sur-8(ku167)* to suppress *let-60(n1046)*. For comparison, *dpy-13 sur-8(ku167) let-60(n1046)/sur-8(ku167) unc-5 let-60(n1046)* aminals were 22% (239) Muv and had 117% (26) average induction.

sur-8 Positively Regulates Ras Pathway Signaling during Vulval Induction

Although *sur-8(ku167)* and *sur-8(ku242)* suppressed the Muv phenotype caused by the activated *let-60 ras(n1046gf)* mutation, neither allele caused an apparent phenotype in a *let-60 ras(+)* background. Mutants displayed wild type vulval induction (Table 2) and appeared to have no additional obvious developmental defects (data not shown). However, the positive role that *sur-8* plays in vulval induction became apparent when examining its effects on Ras-mediated signaling in sensitised genetic backgrounds. First, as described above, *sur-8* mutations could strongly suppress both the Muv and male mating defects caused by the *let-60 ras(n1046gf)* allele. Second, an extrachromosomal array containing multiple copies of the cloned *sur-8* gene enhanced the average vulval induction of both *let-60(n1046gf) /let-60(n1046gf)* homozygous animals (from 154% to 196%) and *let-60(n1046gf) /+* heterozygous animals (Table 1).

Finally, *sur-8 (ku167)* severely affected vulval induction when other *ras* pathway components were compromised. *sur-8(ku167)* dramatically enhanced Vulvaless and larval lethal phenotypes caused by a weak loss-of-function mutation in *mpk-1* (Wu and Han, 1994). *mpk-1(ku1)* mutants alone display nearly wild type vulval induction and only 7% rod-like larval lethality, but *sur-8(ku167)* decreased vulval induction to 0% and increased larval lethality to nearly 100% in the double mutants (Table 2). Because *mpk-1* MAP kinase is a component of the main Ras pathway, this observed genetic interaction suggests that *sur-8* is an important positive regulator of the Ras pathway that functions to increase pathway output. Furthermore, *sur-8(ku167)* also showed strong genetic interactions with a loss-of-function mutation in another regulator of the Ras pathway, *ksr-1* (Sundaram and Han, 1995). *ksr-1(ku68)* mutants alone display wild type vulval induction (100%) and a weak rod-like lethal phenotype (24%). In *sur-8(ku167); ksr-1(ku68)* double mutants, vulval induction was reduced to 4% and the rod-like larval lethality was increased to 85% (Table 2). This strong genetic interaction between *sur-8* and *ksr-1* suggests that while the function of neither gene is normally required for Ras signaling, their functions are collectively essential for Ras signaling.

Table 2. Genetic Interactions between *sur-8* and *ksr-1* or *mpk-1* MAP kinase Mutations

	%Induction[a]								
genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	%Induction	(n)	%Lethal[b] (n)
N2 (wild type)	0	0	100	100	100	0	100	(many)	0 (138)
sur-8(ku167)	0	0	100	100	100	0	100	(28)	0 (244)
sur-8(ku242)	0	0	100	100	100	0	100	( )	0 (347)
mpk-1(ku1)[c]							98	(17)	7 (229)
mpk-1(ku1) sur-8(ku167)[c]	0	0	0	0	0	0	0	(25)	100 (80)
ksr-1(ku68)[d]	0	0	100	100	100	0	100	(15)	24 (257)
sur-8(ku167); ksr-1(ku68)[d]	0	0	0	11	0	0	4	(19)	85 (164)

[a] VPC scored as induced if it adopts a 1° or 2° fate.

[b] percent of animals arresting with an early larval Rod-like phenotype, characterisetic of loss of function of ras pathway mutations

[c] *mpk-1* marked with *dpy-17*. *sur-8* marked with *unc-24*. Dyp Unc self progeny of ku1 dpy-17/+ +; ku167 unc-24 mothers were scored for induction. Dpy Unc animals are almost completely sterile (average brood=4), and all projeny die as early larval Rods.

[d] *ksr-1* marked with *lon-2*. *sur-8* unmarked.

sur-8 Functions Downstream or in Parallel of _ras_ and not Downstream of _raf_

To determine at which step in the linear Ras pathway *sur-8* may function, we performed epistasis analysis with mutations that cause Muv phenotypes. *sur-8* mutations almost completely suppress the Muv phenotype caused by *let-60 ras(n1046gf)*, suggesting that *sur-8* acts downstream of or in parallel to *let-60 ras* (Table 3). We tested whether *sur-8* mutations could suppress the Muv phenotype caused by a loss-of-function mutation in *lin-15, n765*. *lin-15* functions upstream of *ras* at the level of *let-23* RTK to inhibit *let-23* signaling (Ferguson et al., 1987) and encodes two novel proteins that are expressed in the surrounding hypodermis (Clark et al., 1994; Huang et al., 1994). As expected, *sur-8* mutations could suppress the *lin-15* mutant Muv phenotype (Table 3), consistent with *sur-8* functioning downstream of *let-60 ras*. However, this suppression was not complete, possibly due to the inability of *sur-8* mutations to overcome strong pathway activity caused by the *lin-15(n765)* mutation.

In order to define a downstream limit for *sur-8* function, we tested whether a *sur-8* mutation could suppress the Muv phenotype caused by genes acting downstream of *ras*. *lin-1* is a negative regulator of the Ras pathway and encodes a putative transcription factor that by genetic criteria acts downstream of *mpk-1* MAP kinase (Beitel, et al., 1995; Wu et al., 1994; Kornfeld et al., 1995). A loss-of-function mutation of *lin-1, ar147*, causes a 100% Muv phenotype that was not suppressed by *sur-8(ku167)*. Double mutants displayed the same 100% Muv phenotype as the *lin-1* mutants (Table 3), suggesting that *sur-8* does not act downstream and probably acts upstream of *lin-1*.

Genetic studies indicate that *lin-45 raf* acts downstream of *let-60 ras* in the vulval induction pathway (Han et al., 1993). Animals carrying an activated *raf(gf)* transgene under the control of a heat shock promoter displayed a Muv phenotype upon heat shock (Table 3). As expected, the Muv phenotype was completely suppressed by weak mutations in either *mek-2* or *mpk-1* (Table 3), which act downstream of *lin-45 raf* (Kornfeld, 1997; Sundaram and Han, 1996). However, a *sur-8* mutation failed to suppress the Muv phenotype caused by the *raf(gf)* transgene. Heat shocked *raf(gf)* mutants or *sur-8(ku167)*; *raf(gf)* double mutants displayed similar average vulval induction of 116% and 125%, respectively (Table 3), indicating that *sur-8* does not function downstream of *lin-45 raf* in the same linear pathway as *mek-2* and *mpk-1*. Taken together, the epistasis data indicate that *sur-8* acts downstream or in parallel to *let-60 ras* but not downstream of *lin-45 raf*.

Table 3. Epistatic Analysis of *sur-8(ku167)* and Muv Mutants

genotype[a]	%Muv	(n)	%Induction	(n)
+ *let-60(n1046)*	88	(240)	154	(27)
sur-8(ku167) let-60(n1046)	4	(333)	102	(43)
+ *; HSP-raf (gf)*	36	(31)	116	(31)
sur-8(ku167); HSP-raf (gf)	64	(28)	125	(28)
mek-2(ku114); HSP-raf (gf)	0	(25)	97	(25)
mpk-1(ku1); HSP-raf (gf)	0	(28)	99	(28)
+ *; lin-15 (n765)*	98	(214)	190	(24)
sur-8(ku167); lin-15 (n765)	73	(302)	142	(21)
sur-8(ku242); lin-15 (n765)	58	(258)	133	(23)

lin-1(ar147)	+	100	(154)	ND
lin-1(ar147) sur-8(ku167)		100	(184)	ND

[a] for *HSP-raf(gf)* experiments, transgenic animals were heat shocked for 80 minutes at 37°C. for *lin-15* experiments, animals were grown at 19.2°C to reduce expressivity.of the *n756ts* allele.

sur-8 Function is Required during Vulval Induction

Vulval cell fate specification takes place at the end of the L2 stage, after the anchor cell is born and before the VPCs undergo their first division (Kimble, 1981). To determine if *sur-8(+)* activity is required at this stage for proper vulval induction, we assayed the ability of *sur-8(+)* to rescue the suppression phenotype of *sur-8(ku167) let-60(n1046gf)* animals at various stages of development. We generated transgenic *sur-8(ku167) let-60(n1046gf)* animals carrying *sur-8* cDNA (see below) under the control of a heat inducible promoter and subjected them to heat shock at different developmental stages. Control transgenic animals without heat shock displayed a slightly rescued phenotype of 123% vulval induction probably resulting from leaky *sur-8* expression from the heat shock promoter. Animals heat shocked before or during vulval induction (between early L2 and mid L3 stages) displayed a fully rescued phenotype resulting in over 160% induction (Table 4) similar to that observed in *sur-8(ku167) let-60(n1046gf)* mutants carrying a transgene of *sur-8* under control of its own promoter (Table 1). In contrast, animals heat shocked either in L1, before the anchor cell is born, or in L4, after Pn.p cells have executed their fate, displayed only a partially rescued phenotype of 133% or 134% vulval induction (Table 4). The rescuing activity observed in early L2 heat shocked animals is most likely due to SUR-8 protein perdurance. Thus, *sur-8(+)* activity is required before or during the time of vulval cell fate specification for vulval development but is not required at earlier or later times.

Table 4. Recsue of *sur-8(ku167) let-60(n1046)* Phenotype by Human *sur-8* cDNA under Control of a Heat Shock Promoter

extrachromosomal array	induction % P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	total induction	n
vector	0	8	100	100	100	6	103%	38
Ce *sur-8*	61	97	100	100	100	93	184%	42
Hs *sur-8*	16	40	100	100	100	70	142%	25

Transgenic *sur-8(ku167) let-60(n1046)* L2 hermaphrodites were heat shocked for 45 minutes at 37°C and scored for vulval induction at stage L4.

sur-8 Encodes a Novel Leucine-Rich Repeat Protein

We cloned *sur-8* by genetic mapping followed by transformation-rescue (see Experimental Procedures). *sur-8* was mapped to position 1.86 on chromosome IV between the markers *dpy-13* and *unc-5*. Cosmids containing genomic DNA from this region were tested for *sur-8(+)* activity by assaying their ability to revert the Suppressed phenotype of *sur-8(ku167) let-60(n1046gf)* animals back to Muv. A single cosmid, AC7, contained complete rescuing activity as did a 12kb AC7-derived subclone (data not shown). The subclone was predicted to contain a single gene, designated AC7.1 by the *C. elegans* genome sequencing project. A full length 2.1 kb cDNA was identified by screening a mixed stage library (gift from Peter Okkema) using a genomic probe derived

from the predicted AC7.1 gene. Northern blot analysis indicated that this cDNA was the only transcript encoded by *sur-8* (data not shown). We conclude that the gene defined by the isolated cDNA corresponds to *sur-8* because missense mutations from *sur-8* mutant DNA are located in the coding region of the cDNA, and because this cDNA was able to rescue *sur-8* mutants (see below).

The 1.7 kb coding sequence of *sur-8* is predicted to encode a novel 559 amino acid protein containing 18 tandem repeats of the leucine-rich repeat (LRR) motif (amino acids 89-505) (Figure 2). LRRs, characterized by a consensus composed of leucines at invariant positions, are found in a variety of proteins with diverse biological functions and are proposed to mediate protein-protein interactions (Kobe and Deisenhofer, 1994). 15 of the 18 SUR-8 LRRs are 23 amino acids long and form a consensus that is similar to that of yeast adenylate cyclase LRRs (Figure 3). These 23 amino acid LRRs form two tandem clusters of 9 and 6 repeats that are separated by 3 tandem LRRs that are 24 amino acids long and form a distinct consensus with no obvious similarity to other known LRR motifs. SUR-8 contains N-terminal and C-terminal non-LRR flanking sequences of 88 and 53 amino acids, respectively (Figure 2).

We have identified a missense mutation associated with each *sur-8* allele (Figure 2). *sur-8(ku242)* encodes a cysteine 233 to tyrosine substitution in a consensus position within LRR 7. *sur-8(ku167)* encodes a glutamic acid 430 to lysine substitution in a non-consensus position within LRR 15. Both mutations were found to alter amino acids conserved in mammalian *sur-8* homologues (Figure 2 and see below) indicating that these residues may have an evolutionarily conserved function.

***C. elegans sur-8* is Structurally and Functionally Conserved in Mammals.**

An expressed sequence tag (EST) database search revealed several overlapping human and mouse ESTs that shared from 49% to 70% amino acid identity with the non-LRR C-terminal sequences of Ce SUR-8. We used primers derived from a human or mouse EST (GenBank accession numbers W51818 and AA286839, respectively) to amplify the 5' ends of the cDNAs by performing 5' RACE from human brain cDNA or mouse liver cDNA (Clonetech). Sequences from the 5' RACE and EST clones were compiled to generate the full-length (4.1 kb) human and mouse *sur-8* cDNAs. Multi-tissue Northern blot (Clonetech) analysis using a probe derived from the human cDNA revealed that this cDNA corresponded to a single transcript of the predicted size, and the transcript was detected in all tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (data not shown). The predicted proteins encoded by the human and mouse cDNAs are 98% identical at the amino acid level.

Comparison of the amino acid sequences encoded by the mammalian and *C. elegans sur-8* genes revealed significant homology in amino acid sequence and overall protein structure. Mammalian SUR-8 contains exactly the same number of LRRs with the identical length and organization as *C. elegans* SUR-8 LRRs (Figures 2 and 3). The LRR regions share 58% identity and the C-terminal extensions share 76% identity while the N-terminal extensions share very little identity (Figure 2). The 23 amino acid repeats in Hs SUR-8 have the same consensus sequence as their Ce SUR-8 counterparts (Figure 3).

We found that in addition to sharing a high degree of structural homology, the *C. elegans* and human SUR-8 proteins share functional homology. Human *sur-8* cDNA expressed under the control of a heat shock inducible promoter was able to rescue the mutant phenotype of *sur-8(ku167) let-60(n1046gf)* animals (Table 4). Hs *sur*-8 could revert the Suppressed phenotype from 103% induction to 142% induction. Control animals expressing Ce *sur-8* cDNA displayed a fully rescued phenotype with 184%

induction (Table 4). Because Hs *sur-8* could provide *sur-8(+)* activity in *sur-8* mutants, we conclude that Hs *sur-8* is a functional homologue of Ce *sur-8*.

SUR-8 Interacts with LET-60 RAS, but not with a LET-60 RAS Effector Domain Mutant

Yeast adenylate cyclase contains 26 LRRs (Kataoka et al., 1985) which are required for binding to and activation by Ras during vegetative yeast growth (Field et al., 1990; Suzuki et al., 1990). The observation that SUR-8 LRRs form a consensus that is similar to that of yeast adenylate cyclase LRRs led us to speculate that SUR-8 may bind LET-60 Ras. We used the yeast two-hybrid system to test the interaction between SUR-8 and several Ras pathway components. While we failed to detect an interaction between SUR-8 and wild type LIN-45 Raf, MEK-2 MEK, MPK-1 MAP kinase or KSR-1 (data not shown), we detected an interaction with wild type LET-60 Ras, as assayed by the activation of a His reporter (Figures 4 and 5A) and a lacZ reporter (data not shown).

Given that mutations in the LRR regions result in loss of *sur-8* function, we examined the effect of these mutations on LET-60 RAS interaction. Interestingly, while *ku167* E430K had no effect on LET-60 Ras interaction, *ku242* C233Y eliminated detectable interaction with LET-60 Ras (Figure 4A), even though both mutant proteins were expressed at similar levels in yeast (data not shown). The *ku242* C233Y mutation is in LRR 7 of the N-terminal LRR cluster and these data demonstrate that this residue is critical for both SUR-8 function and LET-60 Ras binding.

To define a specific region of SUR-8 involved in LET-60 Ras binding, we tested SUR-8 deletion mutants for LET-60 Ras interaction (Figure 4A). Deletion of the N-terminal 88 amino acid non-LRR region resulted in elimination of LET-60 Ras binding. Similarly, deletion of the C-terminal 53 amino acid non-LRR sequence resulted in reduction of LET-60 Ras binding. In addition, deletion of the last six LRRs, including repeat 15, abolished LET-60 Ras binding. It is likely that N-or C-terminal deletions alter global protein structure that may result in decreased LET-60 Ras interaction.

We next wanted to define a domain of LET-60 Ras required for SUR-8 interaction. We tested interaction of SUR-8(+), and as a control, LIN-45 Raf, with several different LET-60 Ras mutants using the yeast two-hybrid system. Mutations tested included point mutations that cause phenotypes in *C. elegans* (Beitel et al., 1990), deletion mutations, and effector domain mutations (Figure 4B). Point mutations in the effector domain of H-Ras have been shown to abolish binding to several putative Ras binding proteins, including Raf1, PI-3 kinase, and Ral-GDS (Rodriguez-Viciana et al., 1997).

All of the LET-60 Ras loss-of-function mutations tested had no effect on SUR-8 or LIN-45 Raf binding. In addition, the mutation encoded by the gain-of-function allele, *n1046gf* G13E, had no effect on SUR-8 binding or LIN-45 Raf binding. Deletion of the membrane targeting region had only a slight effect on SUR-8 binding or LIN-45 Raf binding. An effector domain double mutation, E37G Y40C, interfered with LIN-45 Raf binding, but had no effect on SUR-8 binding (Figure 4B), suggesting that SUR-8 does not share binding specificity with Raf.

However, we identified one effector domain mutation, P34G, that specifically interfered with SUR-8(+) binding but had no effect on LIN-45 Raf binding. A P34G Ras mutant has previously been shown to bind Raf1 with wild type affinity in vitro, but fails to have transforming activity or neurite outgrowth-inducing activity in vivo (Akasaka et al., 1996). SUR-8 is thus a likely candidate for promoting full Ras activity through binding the Ras effector domain at a site that is distinct from that of Raf.

Hs SUR-8 Interacts with N-Ras and K-Ras 4B but not H-Ras in Vitro

Given the functional and structural homology between Ce *sur-8* and Hs *sur-8*, we were interested in determining whether Hs SUR-8 could bind mammalian Ras. We tested the interaction of Hs SUR-8 with three human Ras family members, N-Ras, K-Ras 4B and H-Ras, in the yeast two-hybrid system. We detected a strong interaction between Hs SUR-8 and two family members, K-Ras and N-Ras but detected only a weak interaction with H-Ras (Figure 5A). As a control for Ras expression, we showed that all three Ras family members interacted strongly with Raf1 (Figure 5A). In addition SUR-8 and Ras displayed cross-species interactions, reinforcing the idea that *sur-8* function may be evolutionarily conserved.

These observations were confirmed by testing in vitro interaction of Hs SUR-8 and Ras family members (Figure 5B). Bacterially expressed GST-Ras fusion proteins were purified and tested for their ability to interact with purified Hs SUR-8 or Raf1. While all three family members bound Raf1 with similar affinities, only N-Ras and K-Ras were capable of binding Hs SUR-8 strongly. However, while Raf1 bound Ras with GTP dependence, Hs SUR-8 showed no GTP dependence for Ras binding. Both GDP and GTP loaded Ras proteins bound Hs SUR-8 with similar affinities. Thus, SUR-8 displayed a differential binding specificity for individual Ras family members that appeared not to depend on the activation state of Ras.

Discussion

In this study, we describe the identification of *sur-8*, a novel regulator of the Ras-mediated signal transduction pathway during vulval induction, and we provide genetic evidence that *sur-8* acts to positively regulate Ras pathway signaling. In addition, we show that *sur-8* encodes a highly conserved, novel, LRR containing protein that binds to a subset of Ras family members in vitro. These findings suggest that *sur-8* defines a regulatory branch point in the Ras pathway, and *sur-8* may either be involved influencing *ras* activity or be a *ras* target.

sur-8 Positively Regulates *let-60 ras* Signaling

Several lines of genetic data indicate that the normal function of *sur-8* is to positively regulate Ras pathway signaling during cell fate specification. First, loss-of-function mutations in *sur-8* can suppress the phenotypes of an activated *let-60 ras* mutation. Second, addition of *sur-8(+)* gene copies from either a duplication or from injected transgenes enhances the Multivulva phenotype caused by the activated *ras* mutation. Third, mutations in *sur-8* dramatically enhance the Vulvaless and larval lethal phenotypes caused by a partial loss-of-function mutation of *mpk-1,* indicating that *sur-8* is required for the maximal strength of signaling activity. Finally, a *sur-8* mutation synergizes with a loss-of-function mutation in *ksr-1*, indicating that *sur-8* and *ksr-1* functions are collectively essential for Ras-mediated signal transduction even when the main pathway is wild type.

Consistent with its role in influencing Ras pathway signaling in vulval development, *sur-8* gene activity is required during the time of vulval induction, but not at earlier or later times (Table 4). However, *sur-8* is likely to regulate *ras*-mediated cell specification events in multiple tissues. Mutations in *sur-8* suppress the male mating defect caused by activated *ras* mutations, dramatically enhance the rod-like larval lethal phenotypes caused by mutations in *ksr-1* and enhance lethality and sterility caused by *mpk-1* mutations. The male tail defects of *let-60 ras* were shown to be the result of mis-

specification of B blast cell fates (Chamberlin and Sternberg, 1994; Yochem et al., 1997). The rod-like larval lethal phenotype of loss-of-function mutations in *let-60 ras* and likely its downstream target genes is caused by mis-specification of the excretory duct cell (Yochem et al., 1997). The sterile phenotype of *let-60 ras* and *mpk-1* mutations is a result of defects in germ cell nuclei exiting from pachytene (Church et al., 1995).

Genetic epistasis experiments demonstrate that mutations in *sur-8* can suppress an activated *ras* allele but not an activated *raf* transgene. This analysis indicates that *sur-8* functions genetically downstream of or in parallel to *let-60 ras* but not downstream of *lin-45 raf*, which is consistent with our molecular analysis indicating that SUR-8 directly interacts with Ras. However, it is unlikely that *sur-8* acts directly between them, because, based on studies with their mammalian homologues (Moodie et al., 1993), *lin-45 raf* is likely to be a direct target of *let-60 ras*. Instead, we favor a model in which *sur-8* functions in a branch point either feeding into or out of the pathway at the level of *lin-45 raf* or *let-60 ras*. *ksr-1* has the same epistatic relationship as *sur-8* with the Ras pathway (M. Sundaram and M. H., unpublished), but because mutations in *ksr-1* synergise with mutations in *sur-8*, we believe that these genes are not acting with each other to stimulate signaling, but rather are acting at distinct points.

Because loss-of-function *sur-8* mutations have no effect on vulval induction in a wild type background, we propose that the normal function of *sur-8* is to increase signaling output of the inductive signal-activated Ras pathway. Alternatively, *sur-8* function may be essential in Ras mediated signaling, but its function is redundant with another gene that performs the same role in Ras-mediated signaling.

SUR-8 is a Structurally and Functionally Highly Conserved, Novel LRR Protein

sur-8 is predicted to encode a novel protein composed largely of leucine-rich repeats. SUR-8 contains 18 tandem LRRs. The 23 amino acid repeats from two clusters of nine and six repeats separated by the three 24 amino acid repeats. We have cloned a human and a mouse SUR-8 homologue, which themselves are greater than 98% identical and share high sequence homology and overall protein organization with Ce SUR-8. In addition to sharing a highly conserved protein structure, we have shown that Hs *sur-8* can complement a Ce *sur-8* mutation (Table 4) indicating that *sur-8* function in Ras signaling is evolutionarily conserved.

Leucine-rich repeats are protein motifs of 20-28 amino acids characterized by a core consensus consisting of invariantly spaced leucines and asparagine (LxxLxLxxN). LRRs have been found in many functionally diverse proteins in a variety of organisms and have been shown to mediate protein-protein interactions (Kobe and Deisenhofer, 1994). The crystal structure of porcine ribonuclease inhibitor, which like SUR-8 is almost completely composed of LRR (Hofsteenge et al., 1988), has been determined (Kobe and Deisenhofer, 1993). It forms a non-globular, horseshoe-like structure with the α-helical portion of each repeat aligned in parallel and exposed to the outer surface and the β-strand portion of each repeat exposed to the inner circumference (Kobe and Deisenhofer, 1994). The mutation encoded by *sur-8(ku167)* is a charge reversal in a negatively charged region predicted to form a β-sheet, while the mutation encoded by *sur-8(ku242)* lies in a predicted α-helical region.

Yeast adenylate cyclase functions to regulate vegetative growth in S. cerevisiae and contains 26 LRRs. In S. cerevisiae, but not in higher eucaryotes or in S. pombe, adenylate cyclase interacts directly with Ras and this binding is required for adenylate cyclase activation and cAMP production. Ras binding is GTP dependent and is disrupted by effector domain mutations, indicating that adenylate cyclase is a Ras effector in S.

cerevisiae. This binding is mediated at least in part by LRRs, since mutations in LRRs disrupt Ras binding (Field et al., 1990; Suzuki et al., 1990). Notably, LRRs are not found in eucaryotic adenylate cyclase, and the consensus formed by the LRR of yeast adenylate cyclase is similar to that formed by the 23 amino acid LRRs of SUR-8 (Figure 3).

SUR-8 may Function through Binding to the Ras Effector Domain

Our findings indicate that both Ce SUR-8 and Hs SUR-8 interact with Ras in the yeast two-hybrid system and in vitro (Figure 5). This interaction is specific to Ras, since no interactions were detected between Ce SUR-8 and other Ras pathway components tested (data not shown). Interaction appears to require cysteine 233 in LRR 7 since mutation of this residue to tyrosine in the *ku242* loss-of-function mutant completely blocks LET-60 Ras interaction (Figure 4A). The correlation between loss of *sur-8* function and loss of LET-60 Ras binding suggests that SUR-8 binding to LET-60 Ras is necessary for optimal *sur-8* function. In contrast, a mutation in glutamic acid 430 in LRR 15 encoded by *ku167* has no effect on LET-60 Ras binding. Because the substitutions encoded by *ku242* and *ku167* are located in a separate LRR clusters, it is tempting to speculate that SUR-8 acts as an adaptor protein, binding Ras through one LRR cluster and binding another unidentified protein through the other. Because global structural integrity is likely required for maximal interaction with LET-60 Ras (Figure 4A) it is likely that additional LRRs are required for LET-60 Ras interaction. Indeed, porcine ribonuclease inhibitor binds to its target, RNase, through several repeats that are distant in the primary structure but that are proximal in the crystal structure (Kobe and Deisenhofer, 1993).

Mutational analysis of LET-60 Ras demonstrates that SUR-8 binding to Ras requires effector domain residues that are distinct from those required by Raf, suggesting that SUR-8 and Raf have different effector domain binding specificities. A P34G mutation blocks interaction of LET-60 Ras with SUR-8 but not with LIN-45 Raf. In contrast, mutation of residues E37 and Y40 interferes with interaction between LET-60 Ras and LIN-45 Raf but not between LET-60 Ras and SUR-8, (Figure 4B). Mutations of residues 37 or 40 in H-Ras disrupt Raf1 binding and Ras function (White et al., 1995). The P34G mutation abolishes the ability of Ras to transform NIH3T3 cells and to induce neurite outgrowth while maintaining Raf binding ability (Akasaka et al., 1996). These observations suggest a second requirement for Ras activation in addition to Raf binding that is possibly dependent on interaction with SUR-8.

In addition to disrupting SUR-8 interaction, the P34G mutation has been shown to disrupt the interaction of yeast adenylate cyclase with Ras (Akasaka et al., 1996). Yeast adenylate cyclase contains 28 tandem LRR, which are required for binding to and activation by Ras. Thus, LRR-mediated interaction with the Ras may be an evolutionarily conserved mechanism for Ras-effector interactions.

Hs SUR-8 Binds a Subset of Ras Family Members

Hs SUR-8 specifically binds to K-Ras 4B and N-Ras, but binds only weakly to H-Ras, in vitro and in the yeast two-hybrid system (Figure 5A and B). The in vitro studies suggest that binding is direct and not mediated through other proteins that may be present in the two-hybrid studies. Given the probability of unique roles for different Ras family members, it is possible that SUR-8 is involved with generating family member specifically. SUR-8 LRRs are similar to those found on Rsp-1, which was identified as a multicopy suppressor of K-*ras* transformed cells (Cutler et al., 1992). Our preliminary data indicate that Rsp-1 indeed competes with SUR-8 for Ras binding (unpublished observations), raising the intriguing possibility that Rsp-1 over-expression inhibits Ras

transformation by blocking a functionally significant SUR-8-Ras interaction. This is consistent with the fact that loss of *sur-8* function suppresses activated *let-60 ras* in *C. elegans*.

Intriguingly, Ras binding to SUR-8 does not appear to be GTP dependent. Hs SUR-8 binds both GTP and GDP bound N-Ras and K-Ras in vitro. In contrast, Raf1 binds all three Ras family members with GTP dependence. One possible model for SUR-8 function is that it is involved in the establishment or maintenance of Ras activity by facilitating binding or activation of Ras effectors, such as Raf. Indeed, the mechanisms by which Raf is activated upon membrane recruitment are poorly understood. Alternatively, SUR-8 may mediate its positive effects on Ras activation by inhibiting the activity of a negative regulator of Ras, such as GTPase activating protein, GAP. Finally, SUR-8 may be an adaptor protein for effectors distinct from Raf that act in a branched pathway. Further molecular and biochemical studies should elucidate the evolutionarily conserved role of SUR-8 function in the Ras-mediated signaling process.

Methods and Procedures:

C. elegans Strains and Phenotypic Analysis

N2 and derivative strains were maintained as described by Brenner (1974) and grown at 20°C unless otherwise indicated. Mutations used are as follows. LG I: *mek-2(ku114)* (Wu et al., 1995). LG III: *mpk-1(ku1)* (Wu and Han, 1994), *dpy-17(e164)* (Brenner, 1974), and *unc-119(ed3)* (Maduro and Pilgrim, 1995). LGIV: *sur-8(ku167), sur-8(ku242), unc-24(e138)* (Brenner, 1974) *unc-5(e53)* (Brenner, 1974), *dpy-13(e184)* (Brenner, 1974), *unc-17(e113)* (Alfonso et al., 1993), *lin-1(ar147)* (Beitel et al., 1995), *lin-45(ku112)* (D. Green and M. H., unpublished), *dpy-20(e1282)* (Hosono et al., 1982), *let-60(n1046)* (Ferguson and Horvitz, 1985), *let-60(sy130)* (Han et al., 1990), *mDf4* (Rogalski and Riddle, 1988) and *mDp1(IV;f)* (T. Rogalski, personal. comm.). LGV: *him-5(e1490)* (Hodgkin et al., 1979). LGX: *lon-2(e678)* (Brenner, 1974), *ksr-1(ku68)* (Sundaram and Han, 1995), *lin-15(n765)* (Ferguson and Horvitz, 1985) and *xol-1(y9)* (Miller et al., 1988).

Multivulva (Muv) and Egg-laying defective (Egl) phenotypes were scored as described previously (Ferguson and Horvitz, 1985). Percent larval lethality (Let) was determined by collecting eggs from gravid hermaphrodites for 1 to 2 hours and examining plates for arrested rod-like larvae. Vulval induction was determined by examining the number and locations of VPC descendant nuclei of early L4 larvae under Nomarski optics as described previously (Han et al., 1990). Average vulval induction was scored as 100% if 3 of 6 VPCs were induced (wild type), 0% if 0 of 6 were induced (Vulvaless) or 200% if six of six VPCs were induced (Muv).

Transgenic *let-60 ras(n1046gf)* Suppressor Screen and Isolation of *sur-8* Mutants

The transgenic strain used to screen for suppressors of *let-60(n1046)* carried the integrated array *kuIs14* which contains the *let-60(n1046)* genomic DNA (pMH132) and *dpy-20(+)* genomic DNA (pMH86) (Sundaram et al., 1996). Transgenic L4 hermaphrodites were mutagenised with 50 mM ethylmethane sulfonate (EMS) (Brenner, 1974) and F1 and F2 self progeny were screened for non-Muv animals. Non-Muv animals that produced nearly all non-Muv progeny (less than 5%) were outcrossed once to *let-60(sy130) dpy-20(1282),* and Dpy non-Muv progeny were outcrossed several times to *let-60(n1046)*. The *let-*

60(n1046); sup strains were mapped using 2- and 3-factor mapping methods (Brenner, 1974). Complementation tests were performed with *mek-2* and *lin-45* alleles.

sur-8(ku167) isolated from this screen was outcrossed 7 times and 3-factor mapped using *dpy-13* and *unc-5* on LGIV. 46 of 52 Dpy non Unc recombinants had the genotype *dpy-13 sur-8(ku167) let-60(n1046)*, and 2 of 18 Unc non Dpy recombinants had the genotype *sur-8(ku167) unc-5 let-60(n1046)* placing *sur-8* at map position *1.72* of LG IV.

sur-8(ku242) was isolated from a non-complementation screen. *unc-24 let-60(n1046); lon-2(e678) xol-1(y9)* or *unc-17(e113) let-60(n1046); lon-2 xol*-1 L4 hermaphrodites were EMS mutagenised and mated to *sur-8(ku167) let-60(n1046); him-5* males. We screened approximately 10,000 haploid genomes. Non-Muv F1 cross progeny were isolated, and those that continued to segregate less than 10% Muv progeny were outcrossed to a *let-60(n1046)* strain. *sur-8(ku242)* was unlinked from the Unc mutations by outcrossing with *let-60(n1046)* and picking suppressed non-Unc recombinants. *sur-8(ku242)* was outcrossed with *let-60(n1046)* an additional 3 times

Because *mDf4* caused no lethality when in trans to *sur-8(ku167)*, the non-complementation screen should not have been biased against isolating null alleles. One probable explanation for the lower frequency at which *sur-8* loss-of-function alleles were isolated is that we restricted the characterization of suppressors to those that suppressed to below 5% Muv, which might be too low to isolate many other *sur-8* mutations.

Dosage Analysis

mDf4 is linked to the semi-dominant *dpy-13* allele, *e184* (Rogalski and Riddle, 1988). For deficiency analysis, *mDf4* was linked *to let-60(n1046)* by selecting Muv semi-Dpy recombinants from *dpy-13 mDf4/unc-5 let-60(n1046)* heterozygotes. The recombinant *dpy-13 mDf4 let-60(n1046)* chromosome was balanced with nT1 and used to do a complementation test with either *unc-5 sur-8(ku167) let-60(n1046)* or *unc-5 let-60(n1046)* by scoring non-Unc, semi-Dpy cross progeny [genotype: *unc-5 +/- sur-8(ku167) let-60(n1046); dpy-13 mDf4 let-60(n1046)*] for percent Muv and percent induction.

mDp1 is a free duplication that covers *unc-17, dpy-13, sur*-8 and *unc-5* but not *let-60.* For the duplication analysis, *dpy-13 unc-5 let-60(n1046) ;mDp1* was constructed and tested for rescue of the Suppressed phenotype by crossing with either *sur-8(ku167) unc-5 let-60(n1046)* or *unc-5 let-60(n1046).* Progeny segregating no Dpy (genotype: *sur-8(ku167) unc-5 let-60 (n1046); mDp1*) were scored for percent Muv and percent vulval induction.

Construction of Double Mutants and Transgenic Strains

Double mutants were constructed using standard genetic methods and markers used are indicated in the Tables. For the *sur-8(ku167); lin-15, sur-8(ku242); lin-15, sur-8(ku167); raf(gf)* double mutants and for *sur-8(ku167)* and *sur-8(ku242)* single mutants, the presence of *sur-8* mutations was confirmed by sequencing the appropriate region of *sur*-8 genomic DNA from each strain.

Because *mpk-1(ku1); unc-24 sur-8(ku167)* double mutants were larval lethal, double homozygotes were derived from mothers that were heterozygous for *sur-8(ku167).* Occasionally, Unc segregants were observed which were examined under Nomarski optics for vulval induction and re-plated to observe progeny. Unc animals either represented escapers, which were 0% induced and had no viable progeny, or represented recombinants which were 100% induced and segregated viable progeny.

Transgenic strains were generated by germline transformation as described previously (Mello et al., 1991). Germline-rescue: cosmids spanning the *sur-8* region were obtained from A. Coulson (Sanger Center). 5ug/ml of single cosmids or subclones were coinjected with 40ug/ml of the *unc-119* transformation marker pDP#MM016 (Maduro and Pilgrim, 1995) into *unc-119; sur-8(ku167) let-60(n1046)* animals and non-Unc stable lines were analyzed. The cosmid AC7 rescued the Suppressed phenotype to between 50% and 100% Muv in four of six stable lines generated. pDS12 contained a 13 kb *PstI-SacII* AC7 sub-fragment cloned into pBluescript (Stratagene) and rescued to 100% Muv in three of three stable lines generated. *kuEx83* is a transgene containing pDS12 (injected at 5 μg/ml) and a *sur-5* promoter*::gfp* reporter construct, pTG96.1 (injected at 100 μg/ml).

raf(gf) epistasis: *kuIs17* is a transgene containing *raf(gf)* (pMS88) and *dpy-20(+)* genomic DNA (pMH86, (Han and Sternberg, 1990)) integrated into genome. pMS88 contains a Drosophila *raf* gain-of-function mutant gene cloned into the HSP16-41 vector pPD49.83 (gift from A. Fire). In this *raf(gf)* gene, the kinase domain of Draf is fused to the transmembrane domain of the Torso receptor (Dickson et al., 1992).

Heat shock rescue: pDS23 and pDS25 contain Ce*sur-8* cDNA and Hs *sur-8* cDNA, respectively, cloned into the *NheI* and *KpnI* sites of pPD49.83. Either pDS23 (10ug/ml), pDS25 (20ug/ml) or pPD49.83 (10ug/ml) was coinjected with pUnc-119 (40ug/ml) into *unc-119 sur-8(ku167) let-60(n1046)* and non-Unc stable lines were analyzed. Three pDS23 and two pDS25 bearing independent lines displayed similar vulval phenotypes upon heat shock.

sur-8 cDNA Cloning and Allele Sequencing

A 2kb *HindIII* genomic subclone containing part of the AC7.1 sequence (pDS7) was used as a probe for a mixed stage Northern, identifying a single band of 2.2 kb. This fragment was used to probe a λgt11 mixed stage *C. elegans* cDNA library (gift P. Okkema). From approximately one million plaques screened, 10 positive clones were isolated and their inserts were PCR amplified. The two largest inserts were sequenced using an ABI automated sequencer and were found to each have a 5' UTR, a single open reading frame and two different poly-adenylated 3' UTRs. A cDNA containing an SL1 spliced leader was identified by performing PCR amplification from an early embryonic cDNA library (gift from P. Okkema) using an SL1 primer and a *sur-8*-specific primer. Full length *sur-8* cDNA contains a 94 nucleotide SL1 spliced 5' UTR, a 1680 nucleotide open reading frame and either a 213 nucleotide or a 359 nucleotide 3' UTR depending on the polyA site used.

Molecular lesions were identified by PCR amplification of genomic DNA from lysates from one to five mutant worms and sequencing purified PCR fragments directly. For each allele, all coding regions were amplified and sequenced using primers flanking exons. The cDNA sequence differs from that predicted for AC7.1 in the positions of four splice junctions. Positions of exons and mutations corresponding to the numbering of cosmid AC7 are as follows. Exon 1, 2255-2437; Exons 2, 3789-3920; Exon 3, 4110-4262; Exon 4, 4510-4726; Exons 5, 5390-5592; Exon 6, 6057-6276; Exon 7, 6648-6998; and Exon 8, 8403-8623. *sur-8(ku167)* and *sur-8(ku242)* contained G to A transitions at positions 6827 and 5402, respectively.

Two-Hybrid Strains and Plasmids

Two-hybrid reporter strains were CG1945 and Y187 (Clonetech). Strains were grown and manipulated according to the manufacturer's protocols. Two-hybrid interactions were tested by mating reporter strains transfected with expression constructs and assaying growth on His- plates followed by assaying β-galactosidase expression. *sur-8* constructs

were expressed as fusion proteins with GAL4 activation domain from pACT2 (Clonetech). Full length and mutant Ce *sur-8* were cloned by PCR from λgt11 clone #10 as *NcoI-BamHI* fragments into *NcoI* and *BamHI* sites of pACT2. Full length Hs *sur-8* was PCR amplified form human brain cDNA (Clonetech) and cloned as *BamHI-XhoI* fragments into *BamHI* and *XhoI* sites of pACT2. pYO3 contains the full length *lin-45 raf* cloned into pACT2 (Y. Suzuki, pers. comm.). Full length Raf1 cloned in pGAD was a gift from M. White.

All *ras* constructs contained a C to S substitution in the CAAX box introduced by the 3' PCR primer and were expressed as GAL4 DNA binding fusion proteins by cloning into the *NdeI-BamHI* sites of pAS2. Full length *K-ras 4B*(C185S) and *N-ras*(C186S) were amplified from human brain cDNA (Clonetech). *H-ras*(V12 C186S) was cloned by amplification from *H-ras*(V12) pGSTag (a gift from K. Guan). pMS104 and pMS105 contain *let-60 ras*(C181S) and *let-60 ras*(G13E, C181S), respectively, cloned into pAS2 (M. Sundaram, pers. comm.). Other *let-60 ras* mutants described were cloned by amplifying *NdeI-BamHI* fragments from pMH2010 (M.H. unpublished) and cloned into pAS2 .

In Vitro Binding

Expression, purification and nucleotide loading of GST-Ras fusion proteins was performed as described previously (Kaelin et al., 1991; Zhang et al., 1995), with the following modifications. The Glutathione Sepharose beads (Pharmacia) were washed two times with loading buffer (50 mM Tris-HCl (pH 7.5), 7.5 mM EDTA, 0.5 mg/ml bovine serum albumen (BSA), 1.0 mM DTT), then 1.0 mM GTPγS or GDPβS (Boehringer Mannheim) was added. After 1 hour of incubation at 37°C, the beads were washed two times with binding buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0 mM DTT, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 25 μM $ZnCl_2$ and 0.2% (w/v) BSA].

pDS42, pDS43, pDS44 and pDS74 contain CAAX box C to S substituted *let-60 ras*, *N-ras*, *K-ras* and *H-ras*, respectively, cloned into *NdeI* and *BamHI* sites of pGSTag (gift from K. Guan) to generate GST fusion proteins. pDS73 and pDS75 contain Hs *sur-8* and *raf1* (1-269), respectively, cloned into the *BamHI* and *KpnI* sites of pQE32 (Qiagen) to generate N-terminal 6xHis tagged fusion proteins. Fresh overnight cultures of *E. coli* DH5α transformed with pDS73 or pDS75 were diluted 1:4 in LB broth containing 100 μg/ml ampicillin and 1 mM IPTG. After 4-7 hours of growth at 37°C, pellets were collected in binding buffer containing 100 μg/ml PMSF and lysed by sonication. Lysate supernatant was incubated overnight at 4°C with 15 μl of Glutathione Sepharose beads bound with GST-Ras (approximately 1 μg/ml) in a volume of 0.2 ml. Beads were washed 4 times with binding buffer, 2 times with binding buffer without BSA, and were prepared for SDS-PAGE (10%) and Western analysis. SUR-8 and Raf1 proteins were detected using an anti 5x-His monoclonal antibody (Qiagen). Ras input was detected by coomassi blue staining.

CONCLUSIONS:

Ras-mediated signal transduction pathways play important roles in controlling cell proliferation, differentiation and migration. Mutations in Ras pathway components are associated with the progression of many different types of cancers, including breast cancer. Understanding the regulation of Ras-mediated signaling will lead to a greater understanding of progression and treatment of cancers resulting from pathway deregulation. We

described here the genetic and molecular characterization of *sur-8*, which both positively regulates *ras*-mediated signaling during vulval cell fate specification in *C. elegans*.

sur-8 is defined by two single loss-of-function alleles, isolated as suppressors of the Multivulva phenotype caused by an activated *let-60 ras* transgene. Genetic characterization of *sur-8* indicates that it plays a non-essential but activating role in *ras* signaling downstream of or in parallel to *let-60 ras* and upstream of *lin-45 raf*.

A *sur-8* mutation shows strong synergy with both *ksr-1* and *sur-1/mpk-1* MAP kinase alleles, suggesting that *sur-8* acts in a branch of the pathway distinct from *sur-6* to regulate *ras* signaling activity. *sur-8* has been cloned and is predicted to encode a novel 559 amino acid protein containing 18 leucine rich repeats (LRR) most similar to those found in yeast adenylate cyclase. *sur-8* interacts directly and specifically with *let-60 ras* in the yeast two hybrid system and in vitro. We have cloned the human homologue of *sur-8* from a human brain library and are currently determining if it shares functional and well as structural homology with *sur-8*.

These results provide evidence that *sur-8* defines a branch point in the ras pathway feeding into or out of the pathway at the level of ras or raf. The observation that *sur-8* binds to *let-60 ras* raises the exciting possibility that *sur-8* defines a second *ras* effector in addition to *lin-45 raf* (see figure below). Atlernitivley, *sur*-8 may funstion to modify the activities of Ras regulators such as GAP or GNRF or may be involvolved in the activation of Raf. Current research efforts focus on further characterizing the molecular mechanisms by which *sur-8* positively regulates ras signaling in hopes of understanding regulation of *ras* signaling in mammalian systems.

REFERENCES:

Akasaka, K., Tamada, M., Wang, F., Kariya, K., Shima, F., Kikuchi, A., Yamamotoo, M., Sshirouzu, M., Yokoyama, S., and Kataoka, T. (1996). Differential structural requirements for interaction of Ras protein with its distinct downstream effectors. J. Biol. Chem. *271*, 5353-5360.

Alfonso, A., Grundahl, K., Duerr, J. S., Han, H. P., and Rand, J. B. (1993). The Caenorhabditis elegans unc-17 gene: A putative vesicular acetylcholine transporter. Science *261*, 617-619.

Barbacid, M. (1987). *ras* genes. Annu. Rev. Biochem. *56*, 779-827.

Beitel, G. J., Clark, S. G., and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. Nature *348*, 503-509.

Beitel, G. J., Tuck, S., Greenwald, I. S., and Horvitz, R. H. (1995). The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes & Development *9*, 3149-3162.

Bos, J. L. (1988). The ras gene family and human carcinogenesis: A review. Cancer Res. *49*, 4682-4689.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71-94.

Chamberlin, H. M., and Sternberg, P. W. (1994). The *lin-3/let-23* pathway mediates inductive signaling during male spicule development in *Caenorhabditis elegans*. Development *120*, 2713-2721.

Chamberlin, H. M., and Sternberg, P. W. (1994). The *lin-3/let-23* pathway mediates inductive signalling during male spicule development in *Caenorhabditis elegans*. Development *120*, 2713-2721.

Church, D. L., Guan, K.-L., and Lambie, E. J. (1995). *mek-2*, *mpk-1/sur-1* and *let-60* ras are required for meiotic cell cycle progression in Caenohabditis elegans. Development *121*, 2525-2535.

Clark, S. G., Lu, X., and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. Genetics *137*, 987-997.

Cutler, M. L., Bassin, R. H., Zanoni, L., and Talbot, N. (1992). Isolation of a novel cDNA capable of suppressing v-Ras transformation. Mol. Cell. Biol. *12*, 3750-3756.

Dickson, B., Sprenger, F., Morrison, D., and Hafen, E. (1992). Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature *360*, 600-603.

Ferguson, E., and Horvitz, H. R. (1985). Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. Genetics ***110***, 17-72.

Ferguson, E. L., Sternberg, P. W., and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. Nature ***326***, 259-267.

Field, J., Xu, H., Michaeli, T., Ballester, R., Sass, P., Wigler, M., and Colicelli, J. (1990). Mutations of the adenylyl cyclase gene that block RAS function in *Saccharomyces cerevisiae*. Science, 464-467.

Furth, M. E., Aldrich, T. H., and Cordon, C. C. (1987). Expression of ras proto-0ncogene proteins in normal human tissues. Oncogene *1*, 47-58.

Gutch, M. J., Flint, A. J., Keller, J., Tonks, N. K., and Hengartner, M. O. (1998). The *Caenorhabditis elegans* SH2 domain-containing protein tyrosine phosphatase PTP-2 participates in signal transduction during oogenesis and vulval development. Genes & Development *12*, 571-585.

Han, M., Aroian, R. V., and Sternberg, P. W. (1990). The *let-60* locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*. Genetics *126*, 899-913.

Han, M., Golden, A., Han, Y., and Sternberg, P. W. (1993). *C. elegans lin-45 raf* gene participates *in let-60 ras*-stimulated vulval differentiation. Nature *363*, 133-140.

Han, M., and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during C. elegans vulval induction, encodes a ras protein. Cell *63*, 921-931.

Hodgkin, J. A., Horvitz, H. R., and Brenner, S. (1979). Nondisjunction mutants of the nematode *Caenorhabditis elegans*. Genetics ***91***, 67-94.

Hofsteenge, J., Kieffer, B., Matthies, R., Hemmings, B. A., and Stone, S. R. (1988). Amino acid sequence of the ribonuclease inhibitor from porcine liver reveals the presence of leucine-rich repeats. Biochemistry *27*, 8537-8544.

Hosono, R., Hirahara, K., Kuno, S., and Kurihara, T. (1982). Mutants of *Caenorhabditis elegans* with dumpy and rounded head phenotype. J. Exp. Zool. *224*, 135-144.

Huang, L. S., Tzou, P., and Sternberg, P. W. (1994). The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. Mol. Biol. Cell *5*, 395-412.

Johnson, L., Greenbaum, D., Chichowski, K., Mercer, K., Murphy, E., Schmitt, E., Bronson, R., Umanoff, H., Edelmann, W., Kucherlapati, R., and Jacks, T. (1997). K-ras is an essential gene in mouse with partial functional overlap with N-ras. Genes Dev. *11*, 2468-2481.

Kaelin, G. W., Pallas, D. C., DeCaprio, J. A., Kaye, F. J., and Livingston, D. M. (1991). Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell *64*, 521-532.

Kataoka, T., Broek, D., and Wigler, M. (1985). DNA sequence and characeritzation of the S. cerevisiae gene encoding adenylate cyclase. Cell *43*, 493-505.

Katz, M. E., and McCormick, F. (1997). Signal transduction from multiple Ras effectors. Current Opinion in Genetics and Development *7*, 75-79.

Kimble, J. (1981). Lineage alterations after ablation of cells in the somatic gonad of *Caenorhabditis elegans*. Dev. Biol. *87*, 286-300.

Kobe, B., and Deisenhofer, J. (1993). Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature *366*, 751-756.

Kobe, B., and Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. *19*, 415-421.

Kornfeld, K. (1997). Vulval development in *Caenorhabditis elegans*. Trends Genet. *13*, 55-61.

Kornfeld, K., Hom, D. B., and Horvitz, H. R. (1995). The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in Caenorhabditis elegans. Cell, 902-913.

Leon, J., Guerrero, I., and Pellicer, A. (1987). Differential expression of the ras gene family in mice. Mol. Cell. Biol *7*, 1535-1540.

Maduro, M., and Pilgrim, D. (1995). Identification and cloning of unc-119, a gene expressed in the *Caenorhabditis elegans* nervous system. Genetics *141*, 977-988.

Marshall, C. J. (1994). MAP kinas kinase kinase, MAP kinase kinase adn MAP kinase. Curr. Opin. Genet. Dev. *4*, 82-89.

McCormick, F. (1993). How receptors turn Ras on. *363*, 15-16.

Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans* after microinjection of DNA into germline cytoplasm: Extrachromosomal maintenance and intergration of transforming sequences. EMBO J. *10*, 3959-3970.

Miller, L. M., Plenefisch, L. P., Casson, L. P., and Meyer, B. J. (1988). *xol-1*: a gene that controls the male modes of both sex determination and X chromosome dosage compensation in *C. elegans*. Cell *55*, 167-183.

Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993). Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. Science *260*, 1658-1661.

Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997). Role of phoshoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by ras. Cell *89*, 457-467.

Rogalski, T. M., and Riddle, D. L. (1988). A *Caenorhabditis elegans* RNA polymerase II gene, *ama-1 IV*, and nearby essential genes. Genetics *118*, 61-74.

Schlessinger, J. (1993). How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. *18*, 273-275.

Sundaram, M., and Han, M. (1995). The *C. elegans* ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell *83*, 889-901.

Sundaram, M., and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. BioEssays *18*, 473-480.

Sundaram, M., Yochem, J., and Han, M. (1996). A Ras-mediated signal transduction pathway is involved in the control of sex myoblast migration in *Caenorhabditis elegans*. Development *122*, 2823-2833.

Suzuki, N., Choe, H., Nishida, N., Yamawaki-Kataoka, Y., Ohnishi, S., Tamaoki, T., and Kataoka, T. (1990). Leucine-rich repeats and carboxyl terminus are required for interaction of yeast adenylate cyclase with RAS proteins. Proc. Natl. Acad. Sci. USA *87*, 8711-8715.

Therrien, M., Chang, H. C., Solomon, N. M., Karim, F. D., Wassarman, D. A., and Rubin, G. M. (1995). KSR, a novel protein kinase required for Ras signal transduction. Cell *83*, 879-887.

Umanoff, H., Edelmann, W., Pellicer, A., and Kucherlapati, R. (1995). The murine N-*ras* gene is not essential for growth and development. Prot. Natl. Acad. Sci. USA *92*, 1709-1713.

Wassarman, D. A., Thierrien, M., and Rubin, G. M. (1995). The Ras signaling pathway in *Drosophila*. Curr. Opin. Gen. Dev. *5*, 44-51.

White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M. H. (1995). Multiple Ras functions can contribute to mammalian cell transformation. Cell *80*, 533-541.

Wu, Y., and Han, M. (1994). Suppression of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. Genes & Development *8*, 147-159.

Wu, Y., Han, M., and Guan, K. L. (1995). MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes & Development *9*, 742-755.

Yochem, J., Sundaram, M., and Han, M. (1997). Ras is required for a limited number of cell fates and not for general proliferation in *Caenorhabditis elegans*. Mol. Cell. Biol. *17*, 2716-2722.

Zhang, X., Marshall, M., and Avruch, J. (1995). Ras-Raf complexes in vitro. Meth. Enzymol. *255*, 323-331.